Evaluation of Prophage Gene Revealed Population Variation of 'Candidatus Liberibacter Asiaticus': Bacterial Pathogen of Citrus Huanglongbing (HLB) in Northern Thailand

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Research Article

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Abstract

'*Candidatus Liberibacter asiaticus*' is a non-culturable bacterial pathogen, the causal agent of Huanglongbing (HLB, yellow shoot disease, also known as citrus greening disease), a highly destructive disease of citrus (Rutaceae). The pathogen is transmitted by the Asian citrus psyllid: *Diaphorina citri* Kuwayama. Recent studies, have shown that the HLB pathogen has two prophages, SC1 that has a lytic cycle and SC2 associated with bacterial virulence. This study aimed to search for SC1 and SC2 prophages of HLB in mandarin orange, sweet orange, bitter orange, kumquat, key lime, citron, caviar lime, kaffir lime, pomelo and orange jasmine from five provinces in Northern Thailand. A total of 216 samples collected from Northern Thailand during 2019 and 2020 were studied. The results revealed that 62.04% (134/216) citrus samples were infected with the '*Ca. L. asiaticus*' the bacterial pathogen associated with citrus HLB. The prophage particles are important genetic elements of bacterial genomes that are involved in lateral gene transfer, pathogenicity, environmental adaptation and interstrain genetic variability. Prophage particles were evaluated in the terminase gene of SC1 and SC2-type prophages. The results of the analysis of the prophage terminase genes of SC1 and SC2 revealed four groups of samples in Northern Thailand. The first group was the population of '*Ca. L. asiaticus*' which was non-prophage; the frequency was 7.46% (10/134) from the total infected citrus samples. The second and third groups contained one prophage sequence with 1.49% (2/134) containing SC1-type prophage and 64.93% (87/134) containing SC2-type prophage. The fourth group contained both prophage sequence with 26.12% (35/134) containing SC1 and SC2-type prophages. Samples of leaves showing various HLB symptoms were collected from infected citrus trees which correlated with the prophages. In the first group (non-prophage) the symptoms were much less severe than other groups which had prophages. On the other hand, the third group containing the SC2-type prophage and fourth group containing SC1 and SC2-type prophages showed the most severe symptoms. The evaluation of the prophage effect on HLB-induced leaf symptoms showed that isolates with the SC2-type prophage particles produced the most severe symptoms.

Introduction

Citrus is one of the most popular fruit and is a major fruit crops grown worldwide (Li et al. 2010; Matheyambath et al. 2016). Belonging to the Rutaceae family, the crop is most extensively cultivated in tropical and sub-tropical regions. The genus *Citrus* consists of approximately 1300 species and some hybrids evolved by crossing between native varieties such as *Citrus reticulata* (mandarin), *C. sinensis* (orange), *C. aurantifolia* (lime), *C. limon* (lemon), and *C. maxima* (pomelo), etc. (Berk 2016). According to the FAO 2017, the annual production of citrus fruits have recorded a brisk growth in the past decades, rising from approximately 30 million tons in 1960 to over 147 million tons in 2017, with *C. sinensis* representing approximately 50% of global citrus production, *C. reticulata* (22%), *C. limon/C. aurantifolia* (11%) and *C. paradisi* (6%). However, *Citrus* spp. has recently been hampered by environmental and disease pressures (Wang et al. 2017).

Huanglongbing (HLB), also known as citrus greening, is the most destructive disease of citrus worldwide.

The pathogen is non-culturable, phloem-limited and associated with three species of α -Proteobacteria: '*Candidatus Liberibacter asiaticus*' (Las), '*Ca. L. africanus*' (Laf) and '*Ca. L. americanus*' (Lam) (Bové 2006). The pathogens are transmitted by psyllids including the Asian citrus psyllid: *Diaphorina citri* Kuwayama, *Cacopsylla* (Psylla) *citrisuga* (Hemiptera: Psyllidae) and African citrus psyllid: *Trioza erytreae* (DelGuercio) (Cen et al. 2012; Gottwald 2007). *Trioza erytreae* was found in Africa (Aubert 1987) and *Diaphorina citri* was found in Asia, North America and South America (Bové J 2006). In Thailand, citrus huanglongbing is the main threat to the citrus industry since this disease first appeared in Thailand in the 1960's. The HLB epidemics caused a significant decline in citrus fruit yield. (Roistacher 1996; Schwarz et al. 1973a; Schwarz et al. 1973b)

Bacteriophages are the most abundant organisms in the biosphere with an estimated 10^{31} particles (Wommack and Colwell 2000). Bacteriophages can multiply in living bacterial cells using bacterial materials and phage enzymes by two mechanisms, firstly, the lytic cycle as phage and secondly, the lysogenic cycle as prophage. Phages or prophages have long been used to distinguish bacterial isolates from different environmental and clinical sources (Grange 1975; Hatfull 2010; Salloum et al. 2011). Prophages are important genetic elements of bacterial genomes which are involved in lateral gene transfer, pathogenicity, environmental adaptations, and interstrain genetic variability. (Liu et al. 2011)

A previous report indicated that two highly related prophages SC1 and SC2, which are circular bacteriophage genomes, are associated with '*Ca. L. asiaticus*'. The SC1 carries suspected lytic cycle genes and was found in nonintegrated tandem repeats; SC1 genes inhibited bacterial growth consistent with holin and endolysin functions. In contrast, SC2 lacks the lytic cycle genes and replicates as a prophage excision plasmid, encodes putative adhesin and peroxidase genes which are involved in lysogenic conversion (Fleites et al. 2014; Zhang et al. 2011).

The prophage lysogenic conversion gene products include extracellular toxins, proteins that alter antigenicity, host effector proteins and enzymes which rescue intracellular survival and proteins that facilitate bacterial attachment to host cells (Boyd and Brüssow 2002)

The conventional polymerase chain reaction (PCR) is a DNA amplification method used to detect huanglongbing infected plants (Jagoueix et al. 1996); it is inexpensive and easy to perform. '*Candidatus Liberibacter asiaticus*' (Las)-specific primers for conventional PCR are applied by using the Las-specific primer set Las606/LSS which is a specific part of the Las 16S ribosomal DNA and is a highly sensitive technique (Fujikawa and Iwanami 2012). The other potential gene locus is the bacteriophage-type DNA polymerase region of the prophage DNA that has played an important role in the evolution of bacterial pathogenicity (Boyd and Brüssow 2002). The terminase prophage gene (representing the corresponding prophage) was indicated to be the SC1-type by the detection of the expected approximately 766 bp DNA amplicon with the specific primer set 766F/766R based on the sequence of gene CLIBASIA_05610 in the genome of '*Ca. L. asiaticus*' strain Psy62 (Liu et al. 2011; Duan et al. 2009). The specific primer set 817F/817R was used to amplify fragments from the prophage similar to the SC2-type from the Florida strain UF506 of '*Ca. L. asiaticus*'. This Florida strain was also shown to carry the SC1-type phage in the

nonintegrated tandem repeats, circular form in inoculated periwinkle (*Catharanthus roseus*) plants (Fu et al. 2015). In this study, population variety among the 'Ca. Liberibacter asiaticus' bacterial pathogen of citrus Huanglongbing disease in Northern Thailand was studied and identified by bacteriophages type. In addition, the effect of bacteriophage on symptoms that occurred on leaves were also studied.

Materials And Methods

HLB sample collection

HLB-symptomatic citrus leaves were collected from five provinces in Northern Thailand consisting of Chiang Mai, Chiang Rai, Lampang, Nan and Sukhothai provinces. Citrus species samples which were studied consisted of *Citrus reticulata* (Mandarin orange), *C. sinensis* (Sweet orange), *C. aurantium* (Bitter orange), *C. japonica* (Kumquat), *C. aurantifolia* (Key lime), *C. medica* L. var. *medica* (Citron), *C. australasica* (Caviar lime), *C. hystrix* (Kaffir lime), *C. maxima* (Pomelo) and *Murraya paniculata* (Orange jasmine). The samples were collected between September 2019 and December 2020. All collected leaf samples were stored at 4 °C at the bacteria laboratory, plant pathology, Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University.

DNA extraction

The DNA was extracted from leaf midribs by the CTAB method that was modified from one previously described by Dellaporta et al (1983). Leaf midrib tissue of citrus (500 mg) was incubated with 4 ml cold grinding buffer for 20 min at 4 °C and then ground finely. The extract was centrifuged at 10,000 g for 5 min at room temperature. The supernatant was transferred to a new tube and centrifuged at 14,000 g for 10 min at room temperature.

The pellets were resuspended in 500 µl of CTAB buffer (cetyltrimethylammonium bromide [CTAB]) and incubated at 60 °C for 30 min, then an equal volume of chloroform/isoamyl alcohol (24:1) was added. The mixture was then centrifuged at 7,000 g for 5 min at room temperature. The aqueous supernatant was transferred to a new tube and combined with an equal volume of isopropanol followed by centrifugation at 14,000 g for 10 min at room temperature. The DNA pellet was washed with 70% ethanol and air dried. The DNA was dissolved in sterile distilled water (15 Ω) and stored at -20 °C.

PCR amplification and sequencing

Amplification of the 16S rDNA region of 'Ca. L. asiaticus' was done by PCR. Briefly, specific primers Las606 (5'-GGAGAGGTGAGTGAATTCCGA-3') reverse primer LSS (5'-ACCCAACATCTAGGTAAAAACC-3'), which was exactly 500 bp upstream of LSS in the Las 16S rDNA sequence were used. (Table 1) (Fujikawa and Iwanami 2012). The prophage gene was detected by PCR with the specific primers 766F (5'-CAATAACAGGACCTCTACGC-3') and 766R (5'-ATTGGAAAGACGACGTTAAA-3') was used to detect the SC1-type prophage, the genomic locus of which was annotated as encoding a phage terminase. (Liu et al. 2011) (Table 1). The primer set 817F (5'-AAGCCTCATAGTTAAAACCA-3') and 817R (5'-ATAAGCC

ATTAGATGATTGGA-3'), was used to amplify fragments from a prophage similar to the SC2-type from the Florida strains (Fu et al. 2015) (Table 1). The PCR reactions were performed using a Gradient Thermal Cycler (A200) in a 50- μ l reaction mixture volume containing 2 μ l of DNA template, 20 μ l each of the forward and reverse primers (10 μ M) and Quick Taq HS DyeMix (TOYOBO CO., LTD.). The thermal cycling condition of primer pair Las606 and LSS were as follows: initial denaturing at 95 °C for 5 min; 40 cycles of denaturing at 94 °C for 30 s, annealing at 62 °C for 45 s, extension at 72 °C for 45 s and a final extension step at 72 °C for 10 min. For primer pair 766F and 766R were: initial denaturing at 94 °C for 5 min; 35 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 90 s and a final extension step at 72 °C for 7 min. Primer pair 817F and 817R were: initial denaturing at 94 °C for 3 min; 35 cycles of denaturing at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 1 min and a final extension step at 72 °C for 10 min. Amplification products were evaluated by electrophoresis in 1.2% agarose gels and visualized by staining with RedSafe™ Nucleic Acid Staining Solution (20,000x) (iNtRON BIOTECHNOLOGY, INC., Korea)

Effect of Las and bacteriophage on HLB symptoms

The potential role of prophage in disease development leading to variations in HLB symptoms in infected citrus leaves were investigated. Hence, evaluation of the symptoms that occurred on citrus leaves infected by '*Ca. L. asiaticus*' induced by non-prophage or various prophages, showed various populations of '*Ca. L. asiaticus*' which correlated with symptom that occurred on citrus leaves. The various symptoms were divided and used to assess the severity according to the population of '*Ca. L. asiaticus*' with or without prophage.

Results

HLB sample collection

The current research conducted a Huanglongbing (HLB) disease survey in citrus orchards and backyards consisting of 216 samples from 79 location in five provinces of Northern Thailand which consisted of Chiang Mai 190 samples, Chiang Rai 3, Lampang 11, Nan 5 and Sukhothai 7. Citrus leaf samples were collected from healthy, early and severe symptomatic trees of HLB which exhibited blotchy mottle, yellowing and lamina yellowing on leaves (Fig.1).

PCR amplification and sequencing

Detection of '*Ca. L. asiaticus*' was based on PCR amplification of an expected approximately 500 bp DNA amplicon with primer set Las606/LSS. The prophage terminase gene, SC1-type prophage was indicated by the expected approximately 766 bp DNA amplicon with primer set 766F/766R and SC2-type prophage was indicated by the expected approximately 817 bp DNA amplicon with primer set 817F/817R (Fig.2). The results revealed that 134 of 216 citrus samples from Northern Thailand were infected with '*Ca. L. asiaticus*'; the frequency of the pathogen was 62.04%. The breakdown of citrus samples infected with '*Ca. L. asiaticus*' were as follows: Chiang Mai province 124 samples, Chiang Rai province 3,

Lampang province 3 and Nan province 4. All PCR-positive samples by the primer set Las606/LSS (37 *C. reticulata*, 1 *C. sinensis*, 54 *C. aurantifolia*, 4 *C. medica* L. var. *Medica*, 19 *C. hystrix* and 19 *C. maxima*) detected the prophage terminase gene of SC1 and SC2-type prophages by 766F/766R and 817F/817R. The SC1 and SC2-type prophage was detected in 37 and 122 samples, respectively. For this result, the population of '*Ca. L. asiaticus*' was divided into four groups. The first group, non-prophage, was found in 10 samples. The frequency was 7.46% (10/134) from the total infected citrus samples. The second group contained SC1-type prophage which was found in 2 samples and the frequency was 1.49% (2/134).

The third group containing the SC2-type prophage was found in 87 samples and the frequency was 64.93% (87/134). The fourth group contained both prophages found in 35 samples and the frequency was 26.12% (35/134). The SC2 was found more than the SC1-type prophage because most of the SC2 had the lysogenic cycle as prophage and appeared to lack lytic cycle genes. In contrast, SC1 carried suspected lytic cycle genes as phage, found in this study at only 27.61% (37/134) (Table 2).

Effect of Las and bacteriophage on HLB symptoms

HLB-infected citrus samples were analyzed and the effect of the prophage terminase gene was compared. The population of '*Ca. L. asiaticus*' revealed four groups in Northern Thailand. The first group was '*Ca. L. asiaticus*' which was non-prophage; the frequency was 7.46% (10/134) from the total infected citrus samples; the leaves mainly appeared normal but some leaves had slight yellowing symptoms. The second contained the SC1-type prophage sequence at 1.49% (2/134); the main leaf symptom was a blotchy mottle. The third contained the SC2-type prophage sequence at 64.93% (87/134); the main leaf symptom was interveinal chlorosis. The fourth group contained both prophage sequences at 26.12% (35/134) containing SC1 and SC2-type prophages; the main leaf symptoms were severe blotchy mottle and interveinal chlorosis. Therefore, the citrus leaves were infected with various bacteriophages that affected the symptoms that occurred. These results indicated that the SC1-type prophage caused less of an effect on citrus because of the lytic cycle genes which inhibited bacterial growth.

On the other hand, the SC2-type prophage lysogenic cycle stimulated the pathogenicity of '*Ca. L. asiaticus*'.

The citrus co-infected with SC1 and SC2-type prophages produced more severe symptoms than the other groups. (Fig. 3)

Discussion

Citrus is one of the most important crops in Northern Thailand including Chiang Mai and Chiang Rai provinces. Epidemics of HLB lead to a significant reduction of citrus fruit yield and the abundance of trees. Therefore, this research evaluated the HLB disease and bacteriophages in '*Ca. L. asiaticus*' by PCR products amplified with specific primers designed to detect '*Ca. L. asiaticus*' and the prophage terminase genes of SC1 and SC2-type prophage. The HLB disease was detected by specific primers Las606/LSS and amplification of the 16S rDNA region of '*Ca. L. asiaticus*' which was exactly 500 bp. The PCR results of these 16S rDNA regions confirmed the presence of the '*Ca. L. asiaticus*' according to study of Fujikawa

and Iwanami (2012). '*Ca. L. asiaticus*' was not detected in *Murraya paniculata* samples collected from Chiang Mai. This result differs with the study of Jantasorn et al. (2012) which reported Citrus Huanglongbing in *Murraya paniculata* and *Diaphorina citri* in Thailand in all samples collected from 14 different provinces.

Previously, the prophage study of Tomimura et al. (2009) revealed three clusters of '*Ca. L. asiaticus*' in Southeast Asia by phylogenetic analysis of the bacteriophage-type DNA polymerase sequences. All Indonesian '*Ca. L. asiaticus*' isolates clustered in one group, The second group consisted of mainly Vietnamese and Taiwanese isolates and the third group consisted of Thai and Vietnamese isolates.

In 2011, Zhang et al. described two largely homologous, circular phage genomes, SC1 and SC2, of Las-infected UF506 isolates. SC1 carried lytic cycle genes which form only in plants and can be found nonintegrated in tandem. SC2 encoded putative adhesin and peroxidase genes which may be involved in lysogenic conversion, and is found integrated in tandem with SC1 in the UF506 chromosome and lacks lytic cycle genes. Both SC1 and SC2 prophages were found in psyllids.

In a later study, Liu et al. (2011) elucidated the sequence of a prophage terminase gene of '*Ca. L. asiaticus*' and this was the first report that described the population variation of '*Ca. L. asiaticus*' in the two geographical regions between Guangdong and Yunnan provinces, China. The results revealed that the frequency of the prophage terminase gene was 15.8% in Guangdong which has an altitude < 500 m and 97.4% in Yunnan at an altitude > 2,000 m. For our study, we followed this report and used primer set 766F/766R to identify prophage terminase gene (CLIBASIA_05610) which indicated the SC1-type prophage in the '*Ca. L. asiaticus*' population.

Subsequently, Zhou et al. (2013) revealed seven new prophage variants resulting from two hyper-variable regions identified by screening clone libraries from infected citrus, periwinkle and psyllids. Interestingly, types A and B share highly conserved sequences and localize with two prophages, FP1 and FP2, respectively.

For the first study of genetic variation of '*Ca. L. asiaticus*' populations in Thailand, Puttamuk et al. (2014) studied two hypervariable effector genes, *lasA_I* and *lasA_{II}*. The '*Ca. L. asiaticus*' infected samples collected from Thailand contained 48.29% of prophage 1 (FP1), 63.26% prophage 2 (FP2) and 19.38% containing both prophages. Noticeably, FP2 was the most often found population of '*Ca. L. asiaticus*' in Thailand

In 2015, Fu et al. Studied anatomical analyses of sweet orange trees infected with '*Ca. L. asiaticus*' by light and electron microscopy. The results showed that the samples infected by prophage and '*Ca. L. asiaticus*' led to phloem plasmodesmata plugging with callose; in some samples the phloem was collapsed and chloroplast structures were deformed. In addition, two prophage genomes in '*Ca. L. asiaticus*' were designated SC1(FP1) and SC2 (FP2) from two hypervariable prophage genes with intragenic tandem repeats. The SC1 was designated by Liu et al. (2011) and SC2 was designated from the Florida strains UF506 '*Ca. L. asiaticus*'

For this study, we followed the report of Liu et al. (2011) and Fu et al. (2015) to detect the prophage terminase genes of SC1(FP1) and SC2 (FP2) of the 'Ca. L. asiaticus' population. These researchers described the variation of a phage DNA polymerase gene, we used two specific primers 766F/766R and 817F/817R for detection of the presence of phage genome related to the SC1 and SC2 type-prophages in citrus samples collected from Northern Thailand. Our results showed that SC1 and SC2 type-prophages were detected, corresponding with Liu et al. (2011) and Fu et al. (2015). Apart from these two known prophages there could have been other unknown types of prophage or no prophage at all. (Fu et al. 2019). Bacteriophages provide insight into a virulence mechanism of 'Ca. L. asiaticus'. Prophages effected the evolution of bacteria by disruption of the gene in which they integrate, the facilitation of homologous recombination and the expression of new functions directly related to virulence (Fortier and Sekulovic 2013). The citrus samples infected with 'Ca. L. asiaticus' showed several symptoms as follows: asymptomatic, slight yellowing, blotchy mottle and interveinal chlorosis. The symptom was dependant on the number of prophages and the type of prophage. Further evaluation of symptom severity on the citrus leaves revealed that the citrus leaves were infected with various bacteriophages such as SC1 and SC2-type prophage leading to more severe symptoms than 'Ca. L. asiaticus' isolates which have only of SC1 or SC2-type prophages. In addition, HLB-infected citrus sample had less severe symptoms or were non-symptomatic because they lacked prophages. For this study, SC2-type prophages were found in many HLB-infected citrus samples as a result of the lysogenic cycle which promotes intracellular survival and proteins that facilitate bacterial attachment to host cells of 'Ca. L. asiaticus' (Boyd and Brüssow 2002) On the other hand, SC1 appeared to have the lytic cycle; SC1 was detected by the primer set 766F/766R nonintegrated in the SC1-type prophage (Zhang et al. 2011)

Declarations

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tables

Table 1 Oligonucleotide primers for polymerase chain reaction amplification used in this study

Primer name	Nucleotide sequences (5'–3')	Amplicon size (bp)	Description	References
16S rDNA		500	Las 16S rDNA	Fujikawa and Iwanami. (2012)
Las606	GGAGAGGTGAGTGG AATTCCGA			
LSS	ACCCAACATCTAGGTAAAAACC			
SC1		766	phage terminase gene (CLIBASIA_05610)	Liu et al. (2011)
766F	CAATAACAGGACCTCTACGC			
766R	ATTGGAAAGACGACGTTAAA			
SC2		817	phage terminase gene	Fu et al. (2015)
817F	AAGCCTCATAGTTAAAACCA			
817R	ATAAGCCATTAGATGATTGGA			

Table 2 Summary of polymerase chain reaction (PCR) detection of ‘*Ca. L. asiaticus*’, SC1 and SC2-type prophages from various citrus plants

Host	Total samples	Number of positive				
		HLB	Non-prophage	SC1	SC2	SC1+SC2
<i>Citrus reticulata</i>	62	37	2	1	27	7
<i>Citrus sinensis</i>	1	1	0	0	1	0
<i>Citrus aurantium</i>	1	0	0	0	0	0
<i>Citrus japonica</i>	1	0	0	0	0	0
<i>Citrus aurantifolia</i>	74	54	4	0	36	14
<i>Citrus medica</i> L. var. <i>medica</i>	4	4	0	0	1	3
<i>Citrus australasica</i>	1	0	0	0	0	0
<i>Citrus hystrix</i>	21	19	1	1	11	6
<i>Citrus maxima</i>	45	19	3	0	11	5
<i>Murraya paniculata</i>	6	0	0	0	0	0
Total	216	134	10	2	87	35

Figures

Figure 1

Huanglongbing disease on citrus leaves samples and vectors. a Mandarin orange leaves (*Citrus reticulata*), b Key lime leaves (*Citrus aurantifolia*), c Kaffir lime leaves (*Citrus hystrix*), d Pomelo leaves (*Citrus maxima*), e Adult Asian citrus psyllid (*Diaphorina citri*) and f The waxy exudate from nymphs (circle)

Figure 2

PCR products amplified a Las positive citrus by using primers LSS/Las606 (500 bp), b SC1-type phage DNA sequences amplified by primers 766F/766R (766 bp), c SC2-type phage DNA sequences amplified by 817F/817R (817 bp) (lane M, 100 bp + 1.5 Kb DNA Ladder as size marker; lane 1, 2, 3 and 4 denote isolates of 'Ca. L. asiaticus' from key lime leaves, mandarin orange leaves, kaffir lime leaves and key lime leaves, respectively.; H healthy mandarin orange; W negative control without DNA (distilled water))

Figure 3

The various Huanglongbing symptoms in citrus plants. a Symptomatic mandarin orange leaves, b Symptomatic Kaffir lime leaves, c Symptomatic Key lime leaves, d Symptomatic Pomelo leaves; 1, leaves were infected with Las; 2, leaves were infected with Las and SC1; 3, leaves were infected with Las and SC2; 4, leaves were infected with Las , SC1 and SC2.